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The goal of this project is to analyze the metastasis of mammary tumors in transgenic mice. This will make use of fluorescently labeled tumor cells to allow in vivo visualization of the tumor cells in the primary tumor as well as in target organs. For the first year of this project we have generated 3 transgenic lines carrying the MMTV-GFP expression construct. We have evaluated GFP fluorescence in one of these lines (founder 25) and are in the process of testing two others. Heterozygotes derived from founder 25 show GFP fluorescence in mammary ducts and are now being crossed with transgenic mice carrying the HER2/neu or polyoma middle T transgene.

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FOREWORD

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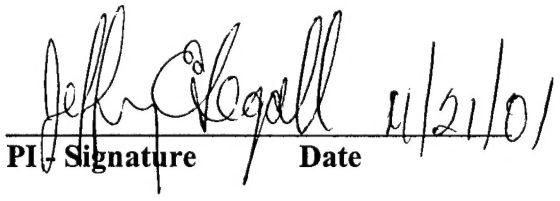

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Introduction

The major life threatening aspect of most cancers involves the metastatic spread of cancer. However, methods for dissecting the process of metastasis need further development in order to be able to determine the functions of specific gene products in metastasis. Transgenic mice expressing or lacking specific cancer related genes have provided a powerful method for evaluating mechanisms of tumor formation. This project will develop new techniques for more detailed dissection of the roles of such gene products in the metastatic process, using GFP (green fluorescent protein)-expressing transgenic mice. In the first technical objective, transgenic mice expressing GFP in the mammary gland have been generated. We will first evaluate an expression construct in which the enhanced GFP cDNA with mammalian codon sequences is driven by the full length MMTV promoter and ends with the SV40 intron and polyadenylation sequence. Transgenic mice which have the highest level of GFP expression in the mammary gland and greatest tissue specificity of expression will be chosen for further work. In the second technical objective, these mice will then be used together with image analysis techniques that we have developed in order to dissect the process of metastasis in more detail. By crossing the GFP-mice with mice containing specific mammary tissue-targeted proteins such as HER2/neu, the contributions of specific oncogenes to the metastatic cascade will be determined. The properties of tumor cells at the border of the tumor, the density of tumor cells in the blood, the kinetics of invasion of target organs, and the distribution of individual tumor cells and metastases in target organs such as the lung can be evaluated using such mice. In the third technical objective, the contribution of other proteins to the various stages of metastasis, such as angiogenesis factors, proteases, cytoskeletal proteins, and adhesion proteins will be determined.

Body

The technical objectives of this project are:

Technical Objective 1: To generate transgenic mice carrying constructs targeting GFP to the mammary gland and to evaluate the specificity of GFP expression.

Technical Objective 2: To cross mice expressing GFP in the mammary gland with mice expressing HER2/neu under the control of the MMTV promoter and utilize the GFP expression to evaluate the metastatic properties of the tumors that develop in these mice.

Technical Objective 3: To evaluate the effects of other transgenes or knockouts on the rate limiting step of metastasis of HER2/neu tumors.

The statement of work is:

STATEMENT OF WORK

Task 1: To generate transgenic mice expressing green fluorescent protein in the mammary gland (months 1 - 12).

- develop expression constructs for mammary targeted expression of GFP (months 1 -3)
- generate founder mice with the aid of the Transgenic Core Facility (months 3 -6)
- identify founders that stably transmit the GFP gene, have high mammary specific expression of GFP (months 6 - 12). This may require testing up to 6

founders with crosses for 2 generations to test transmission and tissue specificity. Up to 240 newborn mice or 72 transgenics may be evaluated.

Task 2. To cross the GFP transgenic mice with HER2/neu mice and evaluate the metastatic cascade for the tumors that develop (months 12 - 24).

- Use at least one test cross to refine assays for analysis of tumor cells in blood and injection of tumor cells for analysis of seeding in lungs (months 13 - 19).Per cross, if two heterozygote trangenics are crossed, to acquire around 20 double transgenics will require around 80 progeny. 10 single transgenic progeny will also be kept to as controls for background effects, although both transgenics are in the FvB background.
- perform the cross and allow tumors to develop as tumors reach .5 - 1 cm, sacrifice mice and analyze the distribution of tumor cells (months 15 - 24).

Task 3. To cross GFP HER2/neu mice with mice defective in matrilysin or gelsolin and evaluate the contributions of these molecules to the various metastatic steps (months 24 -36).

- perform the crosses and allow tumors to develop (months 24 - 30). Per cross, if two heterozygote transgenics are crossed, to acquire around 20 double transgenics will require around 80 progeny. 10 single transgenic progeny will also be kept to as controls for background effects.
- as tumors reach .5 - 1 cm, sacrifice mice and analyze the distribution of tumor cells (months 30 - 36).

Progress for Year 1

In the last report, we reported that we had nearly completed Task 1. We had performed the following:

- develop expression constructs for mammary targeted expression of GFP (months 1 -3) -- we had focussed on an MMTV promoter driving GFP expression.
- generate founder mice with the aid of the Transgenic Core Facility (months 3 -6)
- identify founders that stably transmit the GFP gene, have high mammary specific expression of GFP (months 6 - 12). This may require testing up to 6 founders with crosses for 2 generations to test transmission and tissue specificity. Up to 240 newborn mice or 72 transgenics may be evaluated.

Analysis of the mammary glands using the fluorescence microscope demonstrated that the ducts and potentially alveolar structures are detectable in transgenic mice but not in control nontransgenics. One limitation was the level of GFP expression. We then began generating lines homozygous for the MMTV-GFP construct and began crosses with transgenic animals expressing HER2/neu and polyoma middle T antigen in the mammary gland. The polyoma transgenics generate tumors much more rapidly than the HER2/neu animals and will provide us with more opportunities to refine our methods of imaging tumors in transgenic mice.

By crossing *MMTV-GFP* mice with transgenic mice expressing the polyoma Middle T antigen driven by the *MMTV* promoter (*MMTV-PyMT*) we have produced mouse colonies with GFP expressing tumors (*MMTV-PyMT* X *MMTV-GFP*) of high metastatic grade (Figure 1). Homozygosity for *PyMT* cannot be achieved for female mice in a breeding colony without the use of a foster mother. Since 100% of all heterozygous female mice for *MMTV-PyMT* develop mammary tumors prior to the onset of puberty, which prevents lactation, we have developed a breeding colony from two males homozygous for *MMTV-GFP* and heterozygous for *MMTV-PyMT*. These were subsequently crossed with homozygous females for *MMTV-GFP* to maintain the line. *MMTV-PyMT* induces mammary tumors in 100% of mice by 4 weeks of age and these tumors progress to metastatic tumors between 16 and 22 weeks.

We have also produced mouse colonies with GFP expressing tumors driven by the *neu* oncogene. These differ from the *MMTV- PyMT* animals, since tumor onset is after puberty and the onset of pregnancy enhances tumor growth. Hence, we are able to create a breeding colony of pure homozygous mice for *MMTV-neu* and *MMTV-GFP* (figure 1).

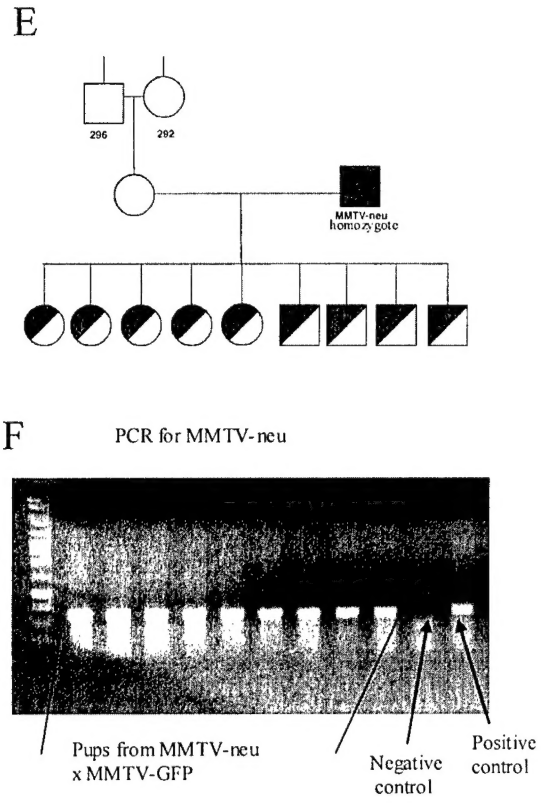

Figure 1: **Genealogies for *MMTV-GFP*, *MMTV-PyMT* x *MMTV-GFP* and *MMTV-neu* x *MMTV-GFP*.** A) Genealogy for MMTV-GFP transgenic mouse shows the development of a homozygous breeding colony. B) A southern blot for *GFP* in pups from a *MMTV-GFP* x FvB cross confirms homozygosity. C) Genealogy for *MMTV-PyMT* x *MMTV-GFP* shows the development of two homozygous GFP x heterozygous PyMT male mice that were used to establish a homozygous GFP breeding colony with males heterozygous for *MMTV-PyMT*. D) PCR confirmation of the DNA for PyMT in these mice. E) Genealogy for *MMTV-neu x MMTV-GFP* shows the development of nine heterozygote pups, male and female, for both neu and GFP that were used to establish a breeding colony of homozygous neu and GFP. F) PCR confirmation of the DNA for neu in these mice.

Analysis of the *MMTV-PyMT* X *MMTV-GFP* mice

The rapid onset of tumors in the *MMTV-PyMT* X *MMTV-GFP* mice has allowed us to analyse enough animals to establish an initial working understanding of the properties of primary tumors in these animals. As noted above, tumors form rapidly compared to those in *MMTV-neu x*

MMTV-GFP. During tumor progression, defined histopathological grades, hyperplasia, adenoma, early- and late- carcinoma, are recapitulated with a high degree of reproducibility. Furthermore, GFP expression does not affect the grade and staging of tumor progression indicating that it is a well behaved neutral marker for following cell behavior *in vivo* (Figure 2). The short latency, high penetrance and reproducible progression of this model makes it ideal for imaging and cell collection studies at defined points of tumor progression.

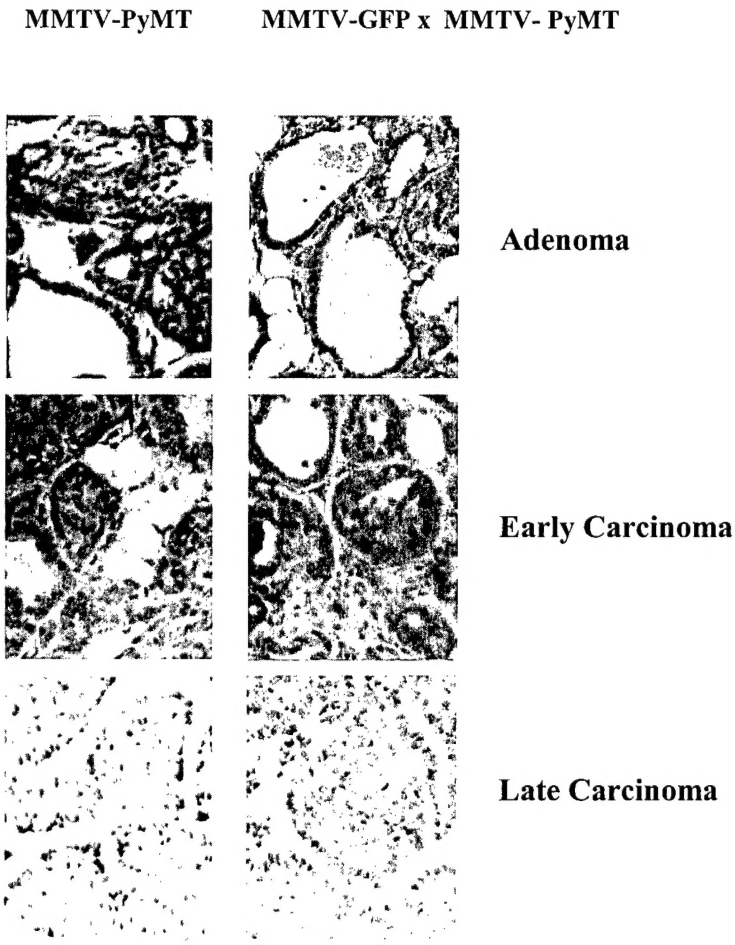

Figure 2: **GFP expression is a neutral marker for use in intravital imaging**. Comparative histology between breast tumors generated in transgenic mice containing only the MMTV-PyMT gene and those containing MMTV-GFP x MMTV-PyMT demonstrates that the mice go through the same tumor stages as those that do not have the GFP gene.

A gallery of images of GFP-expressing carcinoma cells in tumors of *MMTV-GFP* x *MMTV-PyMT* mice is shown in Figure 3. GFP fluorescence is readily observed as is the second harmonic fluorescence of extracellular matrix proteins within the undissected breast tumors of live animals under anesthesia. These results demonstrate the feasibility of creating and imaging the proposed GFP-mice at single cell resolution.

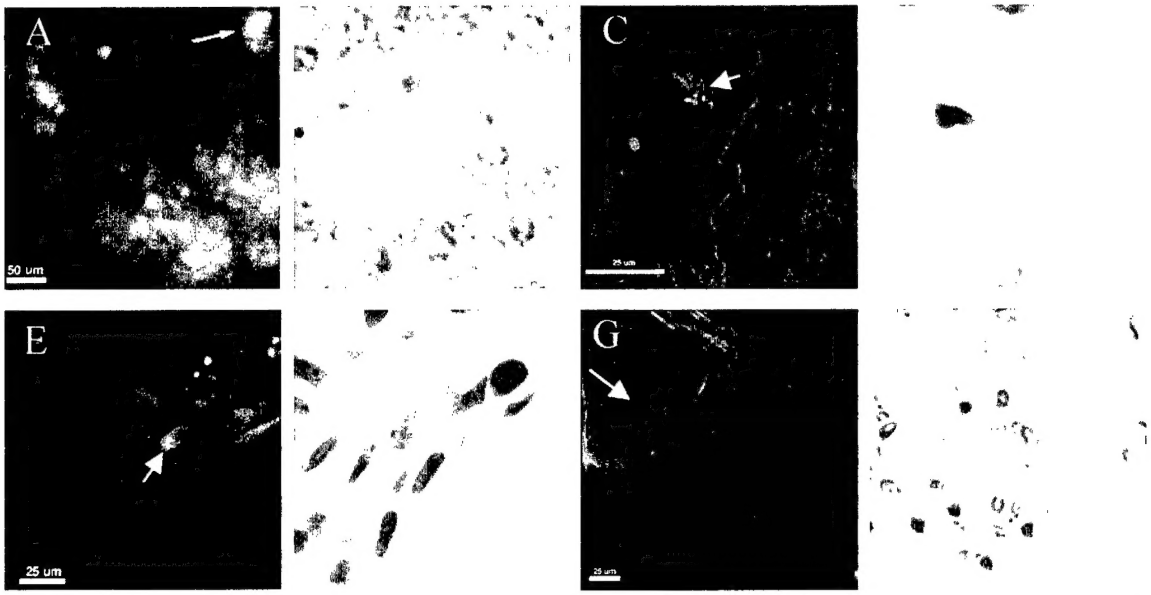

Figure 3: Multiphoton microscopy can be used to image GFP expressing carcinoma cells in tumors of MMTV-GFP x MMTV PyMT transgenic mice. (A, C, E, G) Carcinoma cells expressing GFP (green) were imaged using a multiphoton microscope. Each arrow points to a single cell. GFP-expressing carcinoma cells can be seen interacting with extracellular matrix (blue in C and G). **(B, D, F, H)** Histological sections of areas similar to the areas shown for GFP expressing tumors were used for morphological comparison. Paired images are A/B, C/D, E/F and G/H.

Extracellular matrix encasing clusters of GFP expressing carcinoma cells can also be captured up to a depth of 200 □m (Figure 4). By using the 20 x objective and by taking advantage of the greater depth that the higher wavelength laser emitted by the multi-photon system, we used a step motor to capture images of GFP expressing cells in PyMT generated tumor every 5 □m up to a depth of about 200 □m. Furthermore, by using time-lapsed microscopy, we were also able to capture cell motility (Figure 5). Locomotion of GFP expressing carcinoma cells in a PyMT generated tumor was seen by taking an image every 60 secs for 30 min.

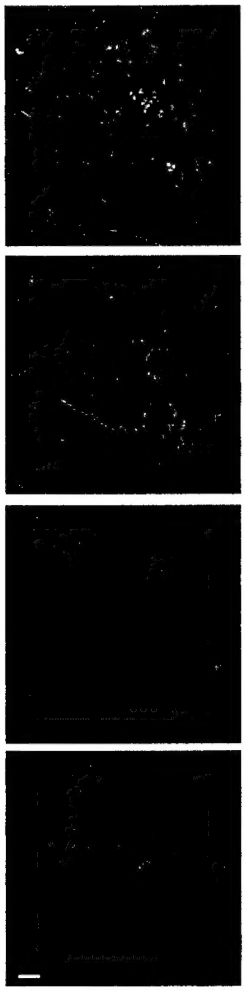

Figure 4: Multiphoton microscopy can be used to image GFP expressing carcinoma cells in tumors from a MMTV-PyMT x MMTV-GFP transgenic mouse up to 200 um into the tissue. A cluster of GFP expressing tumor cells surrounded by matrix, were imaged using multiphoton microscopy. A z-series was taken at 5 μm intervals. The first image shown is 65 μm into the tumor and each step between images represents 15 μm. Scale bar = 25 μm.

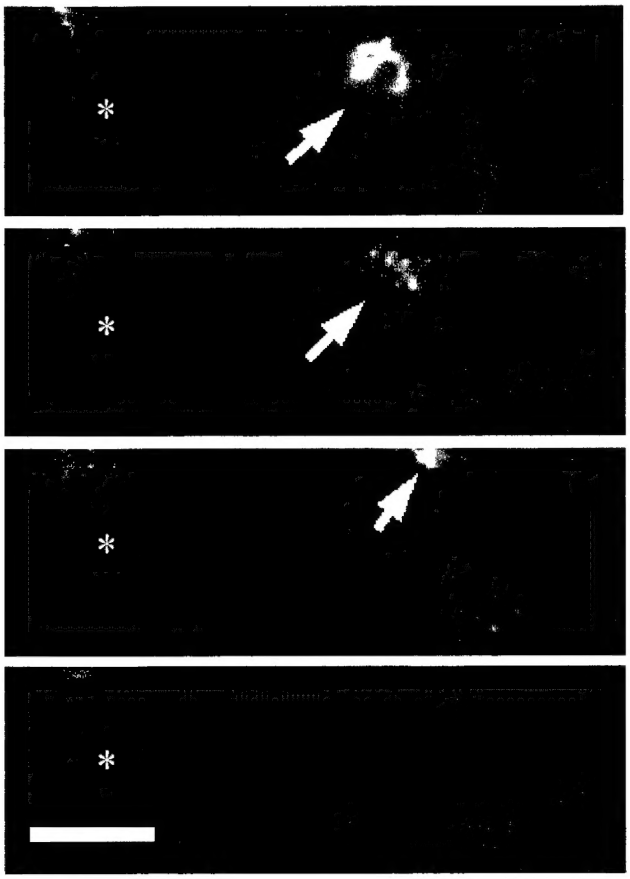

Figure 5: GFP-expressing carcinoma cells can be imaged exhibiting cell motility by multiphoton time-lapsed microscopy. The locomotion of a cell in a MMTV-PyMT x MMTV-GFP generated tumor is seen by *in vivo* multiphoton imaging; capturing images every 60 sec for 30 min. In this figure, the time between sequences is 1 min. The arrow points to the cell exhibiting motility. The asterix marks a stationary cell in the field. Scale bar = 25 μm.

However, there is still variability in intensity of GFP from animal to animal, even in animals homozygous for the MMTV-GFP gene, that makes a detailed analysis problematic. As we continue with our characterization of the MMTV-GFP transgenics crossed to MMTV-HER2 or PyMT animals, we are also exploring alternative methods for expressing GFP in the mammary gland. Thus for task 2 (shown below), we have performed crosses and had tumors develop to the 1 cm stage. However, the fluorescence levels have been too low to allow analysis of tumor cells in blood by GFP fluorescence.

Task 2. To cross the GFP transgenic mice with HER2/neu mice and evaluate the metastatic cascade for the tumors that develop (months 12 - 24).

- Use at least one test cross to refine assays for analysis of tumor cells in blood and injection of tumor cells for analysis of seeding in lungs (months 13 - 19).Per cross, if two heterozygote trangenics are crossed, to acquire around 20 double transgenics will require around 80 progeny. 10 single transgenic progeny will also be kept to as controls for background effects, although both transgenics are in the FvB background.
- perform the cross and allow tumors to develop as tumors reach .5 - 1 cm, sacrifice mice and analyze the distribution of tumor cells (months 15 - 24).

Key Research Accomplishments

- Generation of transgenic mice expressing GFP in the mammary gland under the control of the MMTV LTR.
- Live imaging of tumors of the mammary gland expressing GFP.

Reportable Outcomes

Generation of cell lines expression GFP in the mammary gland and formation of tumors expressing GFP in the mammary gland.

Conclusions

We have partially accomplished Tasks 1 and 2. We have generated transgenic mice expressing GFP in the mammary gland and have crossed them with mice expressing oncogenes. The resulting tumors are fluorescent but the fluorescence levels are marginally adequate. We are pursuing generation of brighter fluorescence while continuing with the lines we have generated.